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Dimethyl sulfoxide inhibits spontaneous diabetes and autoimmune recurrence in non-obese diabetic mice by inducing differentiation of regulatory T cells

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ABSTRACT

Type 1 diabetes mellitus (T1D) is caused by the destruction of insulin-producing β cells in pancreatic islets by autoimmune T cells. Islet transplantation has been established as an effective therapeutic strategy for T1D. However, the survival of islet grafts can be disrupted by recurrent autoimmunity. Dimethyl sulfoxide (DMSO) is a solvent for organic and inorganic substances and an organ-conserving agent used in solid organ transplantations. DMSO also exerts anti-inflammatory, reactive oxygen species scavenger and immunomodulatory effects and therefore exhibits therapeutic potential for the treatment of several human inflammatory diseases. In this study, we investigated the therapeutic potential of DMSO in the inhibition of autoimmunity. We treated an animal model of islet transplantation (NOD mice) with DMSO. The survival of the syngeneic islet grafts was significantly prolonged. The population numbers of CD8, DC and Th1 cells were decreased, and regulatory T (Treg) cell numbers were increased in recipients. The expression levels of IFN-γ and proliferation of T cells were also reduced following DMSO treatment. Furthermore, the differentiation of Treg cells from naive CD4 T cells was significantly increased in the *in vitro* study. Our results demonstrate for the first time that *in vivo* DMSO treatment suppresses spontaneous diabetes and autoimmune recurrence in NOD mice by inhibiting the Th1 immune response and inducing the differentiation of Treg cells.

Introduction

Autoimmune diabetes, formally referred to as type 1 diabetes, results from the destruction of insulin-producing β cells in the islet of the pancreas and has been identified as a T cell-mediated autoimmune disease (Kawasaki et al., 2004). The development of T1D is usually diagnosed in young patients; thus, this disease is also termed juvenile-onset diabetes or childhood-onset diabetes. T1D only exhibits 30–50% concordance in monozygotic twins (Barnett et al., 1981), thereby suggesting that both genetic predisposition and environmental factors contribute to the pathogenesis of diabetes (Kaufman et al., 1992; Abdeen et al., 2007; Nejentsev et al., 2007). The NOD mouse model is frequently used for T1D studies. This mouse model spontaneously develops T cell-dependent β cell destruction resembling human T1D and serves as an animal model for this autoimmune disease (Aoki et al., 2005).

The classical therapeutic strategy for patients with T1D is the administration of insulin injections to maintain normal levels of blood glucose. However, this approach is unable to provide real-time blood glucose modulation and is ineffective in maintaining stable blood glucose levels, which frequently leads to clinical complications, such as retinopathy, nephropathy, neuropathy and macrovascular disease (O'Brien and Corrall, 1988). Maintaining stable glucose levels is important to prevent the development of secondary complications in T1D.

Islet transplantation has been reported as an effective strategy to accomplish insulin independence, normoglycemia and long-term hemostasis of blood glucose in T1D patients (Gaglia et al., 2005). Moreover, islet transplantation is relatively simple to administer, as it does not

Abbreviations: T1D, type 1 diabetes mellitus; NOD mice, non-obese diabetic mice; DMSO, dimethyl sulfoxide; Treg, regulatory T cell; DCs, dendritic cells; IFN, interferon; IL, interleukin

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require major surgical procedures. The procedure can perform on an out-patient basis under local anesthesia and can be repeated several times without major discomfort to the patient (Bottino et al., 2002). Islet transplantation achieves nearly perfect blood glucose monitoring and modulation in T1D patients (Gaglia et al., 2005). However, islet grafts have been shown to be destroyed due to allogeneic graft rejection and autoimmune recurrence (Balamurugan et al., 2006). Autoreactive T cells harbor the memory of β cells and are responsible for this autoimmune recurrence. Islet grafts in NOD mice frequently undergo early graft failure because of the immediate destruction of the graft before immunological graft rejection (Gysemans et al., 2000; Okitsu et al., 2001). Young et al. (2004) have also demonstrated that transplanted islet grafts in NOD mice are susceptible to recurrent autoimmunity. Previous studies have reported that human islets from genetically identical twins (Sibley et al., 1985) or cadaver donors (Tyden et al., 1996) were destroyed by recurrent autoimmunity. Therefore, the establishment of a strategy to suppress autoimmune recurrence is critical for islet transplantation in patients with T1D.

Dimethyl sulfoxide (DMSO) is a colorless, hygroscopic liquid chemical that is a powerful solvent for organic or inorganic substances. It is used for a variety of laboratory and clinical purposes. DMSO is frequently used as a solvent in biological studies and a vehicle for drug therapy. Moreover, DMSO has been applied for therapeutic purposes in several clinical diseases. It has been approved by the United States Food and Drug Administration for the treatment of interstitial cystitis by intravesical instillation (Kato et al., 2000; Santos et al., 2003). It also been used to treat localized amyloidosis and improves renal function and proteinuria in renal amyloidosis patients caused by Crohn's disease (Iwakiri et al., 1999; Kato et al., 2000; Amemori et al., 2006). Furthermore, DMSO is a hydrogen-bond disrupter, hydroxyl radical scavenger and cryoprotectant (Amemori et al., 2006). Due to anti-inflammatory properties of DMSO, it has been justified for the treatment of inflammatory diseases (e.g., scleroderma, osteoarthritis, and rheumatoid arthritis) (Kato et al., 2000; Santos et al., 2003), gastrointestinal diseases (Salim, 1991a,b, 1992), some manifestations of amyloidosis (Morassi et al., 1989; Iwasaki et al., 1994; McCammon et al., 1998; Ozkaya-Bayazit et al., 1998), brain edema (Ikeda and Long, 1990) and chronic prostatitis (Shirley et al., 1978).

The immunomodulatory function of DMSO has recently been described. Kloverpris et al. (2010) have reported that proliferative and secretion of inflammatory cytokines in human CD4 and CD8 T cells were reduced following treatment in 2–3% DMSO solution for 7 days. Lin et al. (1995) have demonstrated that a 4-day treatment with DMSO (>2%) induces apoptosis in Burkitt Lymphoma cells. Given these immunomodulation and anti-inflammatory effects of DMSO, we further investigated whether DMSO treatment prevents the onset of spontaneous diabetes and prolongs the survival of islet grafts in a syngeneic islet transplantation model. Our results demonstrate that DMSO treatment significantly prevented the onset of diabetes and prolonged islet graft survival in NOD mice. To elucidate the mechanisms of this protective effect, we investigated the influence of DMSO on immune cells with regard to cytokine profiles and proliferation of T cells. DMSO treatment reduced the proportion of cytotoxic T cells and DCs and increased the proportion of Treg cells in the spleens of NOD recipients. We further demonstrated that DMSO increases the differentiation of Treg cells from the naive CD4 T cells of NOD mice. Overall, DMSO treatment reduced diabetic incidence in NOD mice and prolonged the survival of syngeneic islet grafts. This protective effect is, at least partly, associated with increased differentiation of Treg cells in the modulation of immune cell effector functions.

Materials and methods

Animals. Inbred NOD/Sytwu mice (K^d, D^b, L^d, $I\text{-}A^{g7}$) and NOD/scid mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA) and subsequently bred at the animal center of the National Defense Medical Center in Taipei, Taiwan, under specific pathogen-free conditions. The spontaneous incidence of diabetes in the colony was 80–90% in females and 20–30% in males by 30 weeks of age.

Islet transplantation. Newly diagnosed NOD female mice, with blood glucose levels between 300 and 500 mg/dL for two consecutive days, were used as recipients for islet transplantation as previous described (Lin et al., 2009). The syngeneic islets were isolated from 5- to 8-week-old non-diabetic male NOD mice. The onset of autoimmune diabetes in male NOD mice is later than female; therefore, we can isolate more intact islets form male NOD mice by this age. Islets measuring between 75 and 150 μm in diameters were selected by hand. Islets (700) were transplanted into the kidney subcapsular space of the recipients. Blood glucose levels lower than 200 mg/dL on the first and second day after islet transplantation were considered to represent a successful transplantation.

Blood glucose monitoring. Blood glucose was monitored daily with a blood glucose test strip (MediSense Optium Xceed, Abbott Diabetes Care Inc., Alameda, CA, USA). Graft rejection was defined as blood glucose levels higher than 300 mg/dL for 2 consecutive days.

Histological analysis. Kidneys were harvested from NOD recipients and then embedded in OCT. Sections (4 μm in thickness) were cut and stained with hematoxylin and eosin (H&E) staining and then analyzed via light microscopy.

Insulin secretion test. Islets were isolated from untreated control mice, PBS-treated mice, and DMSO-treated mice. Fifteen islets were incubated in F12K medium (Gibco, Auckland, NZ) containing 16.7 mM or 2.8 mM glucose for 1 h in Transwell plates. At the end of incubation period, islets were removed and the secreted insulin in the medium was measured with a mouse insulin ELISA kit (Mercodia, Uppsala, Sweden). The stimulation index was calculated with the ratio of insulin secreted at 16.7 and 2.8 mM glucose incubated for 1 h.

T cell proliferation. Splenocytes isolated from female NOD mice were treated with Tris-buffered ammonium chloride to eliminate erythrocytes. After washing, cells were resuspended at a concentration of 5×10^6 cells/mL in RPMI1640 medium supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin. The cells were stimulated with plate-coated anti-CD3 antibody (Clone 145-2C11; BD Biosciences Pharmingen, San Jose, CA, USA), or concanavalin A (Con A; Sigma-Aldrich, Saint Louis, MO, USA). When stimulated with islet antigen, the cells were resuspended in serum-free RPMI-1640 supplemented with 100 U/mL penicillin G, 0.1 mg/mL streptomycin, 0.1 mM MEM non-essential amino acids (NEA, GIBCO Invitrogen, Carlsbad, CA, USA) and 50 μM 2-mercaptothanol (2-ME; Sigma-Aldrich, Saint Louis, MO, USA). After 54 h, the cultured cells were pulsed with 1 μCi of ^{3}H-methyl thymidine (PerkinElmer, Waltham, MA, USA). The plates were then harvested onto a UniFilter-96, GF/C (PerkinElmer) at 72 h. The incorporated ^{3}H-methyl thymidine was measured at 72 h with a TopCount liquid scintillation counter (Packard Instrument Co., Meriden, CT, USA). The level of [^{3}H]-thymidine incorporation for each group was normalized to that of the unstimulated control, thereby producing a ratio referred to here as the stimulation index (S.I.).

Flow cytometry. Lymphocytes were harvested from spleen stained with allophycocyanin-conjugated anti-mouse CD4 (clone GK1.5), phycoerythrin (PE)-conjugated anti-mouse CD8α (clone 53-6.7), fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD19 (clone 1D3), and PE-conjugated anti-mouse CD25 (clone PC61) (BD Pharmingen) for 30 min at 4 °C. For Foxp3 staining, the cells stained with surface molecules were fixed and permeabilized overnight with Fixation/Permeabilization working solution (eBioscience Inc., San Diego, CA, USA).

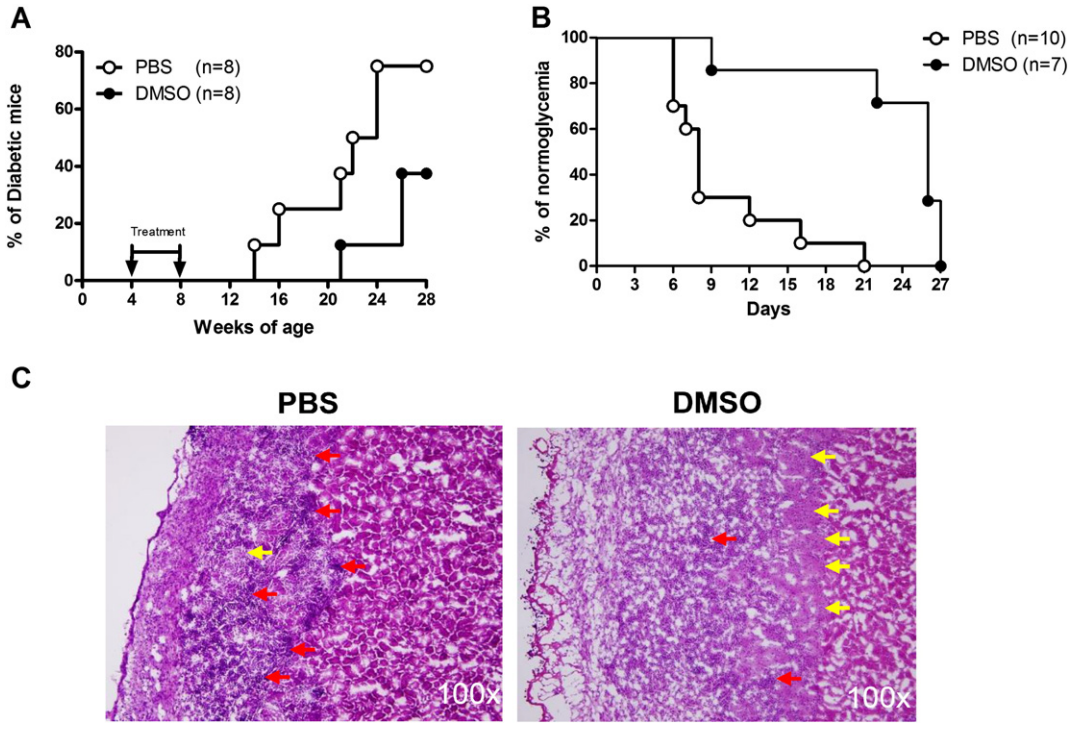

Fig. 1. DMSO treatment delays spontaneous diabetic frequency and prolongs islet graft survival in the syngeneic islet transplantation of NOD mice. (A) Spontaneous diabetes in DMSO-treated NOD mice or PBS-treated NOD mice was monitored via weekly measurement of glycosuria. Diabetic frequency was significantly reduced in the DMSO-treated group ($p < 0.05$). (B) Syngeneic islet grafts in PBS-treated mice were destroyed by day 20 post-transplantation (mean survival 8.8 ± 5.05 days, $n = 10$). The survival time of islet grafts in DMSO-treated mice was prolonged for up to 26 days (mean survival 22.29 ± 8.35 days, $n = 7$, $p < 0.001$ compared with PBS-treated groups). Data are presented as the mean ± SD. (C) The number of infiltrating lymphocytes in islet grafts was lower in the DMSO-treated group at day 10 after transplantation.

After fixation and permeabilization, the cells were stained with FITC-conjugated anti-Foxp3 (clone FJK-16S) (eBioscience Inc.). For intracellular cytokine staining, the cells were stimulated for 4–6 h with 20 ng/mL phorbol 12-myristate 13-acetate and 1 μM ionomycin in the presence of 4 μM monensin. The eBioscience fixation and permeabilization kit was used for intracellular cytokine staining. Stimulated cells were stained with antibody to surface CD4 (RM4-5, APC-conjugated, 0.25 μL/test) on ice for 25–30 minutes (in the dark), washed with 1 mL of FACS buffer (PBS containing 0.5% FBS), and fixed with 0.1 mL of IC Fixation Buffer (eBioscience Inc.) for 20 minutes at ambient temperature (in the dark). After washing with 1 mL of permeabilization buffer (eBioscience Inc.), the cells were resuspended in 80 μL of permeabilization buffer and incubated at ambient temperature for 10 minutes. The cells were then stained with FITC-conjugated anti-IL-10 antibody (clone JES5-16E3) (eBioscience Inc.) at ambient temperature for 20 minutes. Flow cytometric analysis was performed with a FACS Calibur (BD Pharmingen) and CellQuest software (Becton Dickinson, San Jose, CA, USA).

Quantitative real-time polymerase chain reaction (PCR). Total RNA was prepared from splenocytes using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's protocols. Briefly, 2×10^6 splenocytes were suspended in 1 mL of TRIzol reagent and incubated at ambient temperature for 3 minutes. After adding 0.2 mL of chloroform, the tube was agitated by hand for 15 seconds and then incubated at ambient temperature for 2–3 minutes. The suspension was centrifuged at 12,000*g* for 15 minutes at 4 °C, and the aqueous phase was then transferred to a new Eppendorf tube. After adding 0.5 mL of isopropyl alcohol and incubating the suspension at ambient temperature for 10 minutes, the RNA pellet was precipitated via centrifugation at 12,000*g* for 10 minutes at 4 °C. The RNA pellet was subsequently washed with 1 mL of 75% ethanol and centrifuged again at 7500*g* for 5 minutes at 4 °C. The RNA pellet was dried and then dissolved in an appropriate volume of 0.1% DEPC-treated sterile water. Next, 5 μg of total RNA was reverse-transcribed with oligo dT and SuperScript III Reverse Transcriptase (Invitrogen) using oligo $(dT)_{18}$ primers. Real-time PCR was performed using an iCycler (Bio-Rad, Hercules, CA, USA) with iQ SYBR Green PCR SuperMix (Bio-Rad). The threshold cycle (Ct) value was defined as the cycle number at which the fluorescence crossed a fixed threshold above the baseline. For relative quantification, fold changes were measured using the ΔΔCt method. For each sample, the Ct value of cytokine mRNA was measured and compared with the *HPRT* endogenous control as ΔCt ($\Delta Ct = Ct_{cytokine} - Ct_{HPRT}$). The cytokine mRNA fold change in experimental samples relative to control samples was determined using $2^{-\Delta\Delta Ct}$, in which $\Delta\Delta Ct = \Delta Ct_{experiment} - \Delta Ct_{control}$.

In vitro Treg cell differentiation. Naive $CD4^+$ T cells were harvested and sorted from the splenocytes of NOD mice and then cultured for 1 day with human IL-2 cytokine (5 ng/mL). These naive $CD4^+$ T cells were cultured with PBS, 0.5% DMSO, or 1% DMSO for 0, 6 or 24 h. CD4 CD25 foxp3 cells were then measured from these naive $CD4^+$ T cells via flow cytometry.

Western blots. Cells were lysed with 10 mM Tris, 1 mM EDTA, 10 mM KCl and 0.3% Triton, pH 7.9. Proteins were separated on 12% SDS–polyacrylamide gels and electrophoretically transferred to immobilon-P transfer membranes (Millipore, USA). The membranes were blocked with 5% skimmed milk and then incubated with the primary antibodies at 4 °C overnight in TBS-T (10 mM Tris HCl pH 8.0, 150 mM NaCl, and 0.1% Tween-20) followed by a 2 h incubation with peroxidase conjugated secondary antibodies. The protein signals were visualized using an ECL kit (Amersham, Little Chalfont, UK). The following primary antibodies were used: STAT5 (GeneTex, Hsinchu City, Taiwan), phosphorylated STAT5 (GeneTex) and β actin (GeneTex).

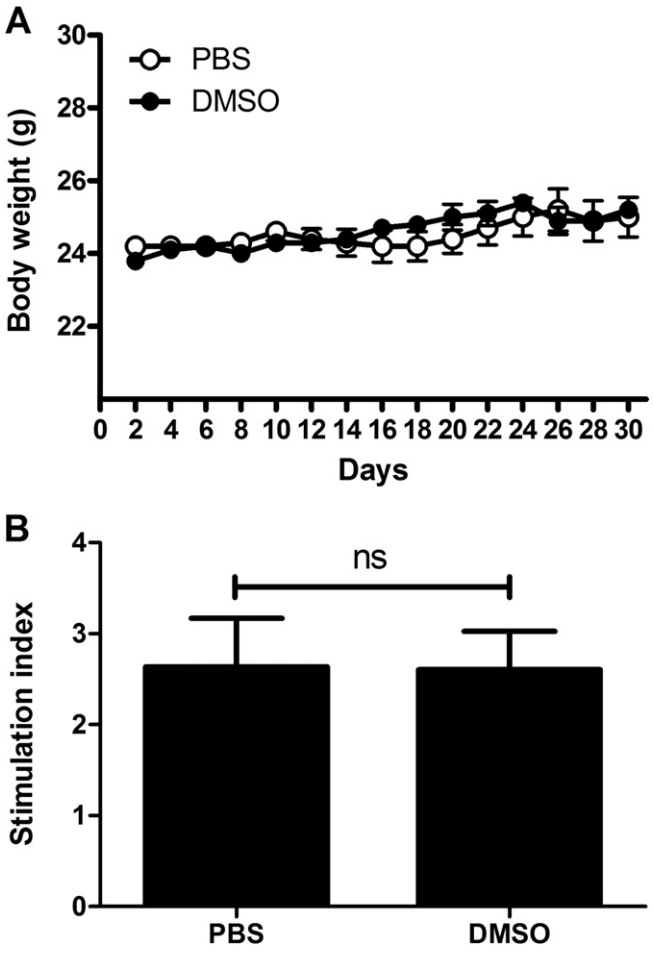

Fig. 2. DMSO treatment does not affect body weight and insulin secretion in NOD recipients. (A) There were no significant differences in the body weight of PBS-treated and DMSO-treated mice ($p > 0.05$, $n = 3$). (B) Islets isolated from PBS- and DMSO-treated mice were stimulated with 2.8 mM or 16.7 mM glucose for 1 h. The stimulation index was calculated with the ratio of insulin secreted at 16.7 and 2.8 mM glucose ($n = 5$). No significant differences between PBS-treated and DMSO-treated groups were observed ($p > 0.05$). The data are expressed as the mean ± SEM ($n = 6$).

Statistical analysis. The data were presented as the mean ± SD or SEM. The significance of islet graft survival time between the PBS-treated and DMSO-treated groups was determined via Kaplan-Meier survival analysis. The significance of diabetic frequency between the PBS-treated and DMSO-treated NOD mice was also determined via Kaplan-Meier survival analysis. For the remaining experiments, *P* values were calculated using the two-tailed Student's *t* test or ANOVA test. Differences are considered significant at $P < 0.05$.

Results

DMSO treatment delayed the onset of autoimmune diabetes and prolonged the survival of syngeneic islet grafts after islet transplantation

To evaluate the protective effect of DMSO in autoimmune diabetes, the 4-week-old female NOD mice were treated with DMSO (0.22 g/kg/day) at 2-day intervals for 4 weeks (16 doses in all for long term therapy). The incidence of spontaneous autoimmune diabetes was significantly lower and the onset of disease was delayed in the DMSO-treated group (Fig. 1A). To investigate whether DMSO treatment protects β cells from autoimmune recurrence, we performed syngeneic islet transplantation. We isolated islets from male NOD mice (age: <8 weeks) and implanted these islets into the left kidney capsule of newly diabetic female NOD recipients. NOD recipients were treated with DMSO (0.22 g/kg/day) daily for a total of 9 days. The treatment was initiated at 1 day prior to islet transplantation (day −1). The entire treatment process was carried out until day 7 post islet transplantation (from day −1 to day 7). In most PBS control recipients, hyperglycemia recurred within 11 days after transplantation, and the mean graft survival time was 8.8 days. In contrast, the islet grafts in most DMSO treated recipients survive up to 20 days, and the mean graft survival time was 22.29 days (Fig. 1B). The biopsy of islet grafts at day 10 after transplantation exhibited low leukocyte infiltration, and more intact islets were observed in the grafted area of the DMSO-treated NOD recipients compared with PBS controls (Fig. 1C). These data indicate that DMSO treatment not only prevents the onset of spontaneous autoimmune diabetes, but it also prolongs the survival of islet grafts after syngeneic islet transplantation.

DMSO treatment did not affect body weight and insulin secretion in NOD mice

To evaluate the effect of DMSO treatment on the general physiology of diabetic NOD mice, we treated newly diabetic NOD mice with DMSO at 0.22 g/kg/day for 9 days and monitored body weight daily. The mean body weight of female diabetic NOD mice showed no apparent difference between DMSO-treated or PBS-treated controls (Fig. 2A). To investigate whether the effect of DMSO on the prolonged survival of islet grafts was caused by increased insulin production, we examined insulin secretion in islets from PBS- or DMSO-treated NOD mice. The levels of insulin secretion from islets incubated in medium with high or low concentrations of glucose displayed no significant difference between control and DMSO-treated groups. There was also no significant difference in the stimulation index between these 2 groups (Fig. 2B). These results indicate that DMSO treatment did not influence body weight in NOD mice or the production of insulin in islets.

DMSO treatment reduced Th1 immune response and increased the population of regulatory T cells

To evaluate the modulatory effect of DMSO treatment on T cells, we analyzed the proportion of different T cell subsets in the spleens at day 8 after islet transplantation in NOD recipients treated with DMSO (at 0.22 g/kg/day for 9 days). No significant difference was found in the percentage of CD4 T cells in the spleens of experimental mice (Fig. 3A). In contrast, the percentage of CD8 T cells was significantly decreased in the DMSO-treated group compared with the PBS-treated group (Fig. 3B, $P < 0.05$). Moreover, the population of CD11c dendritic cells (DCs) was also significantly decreased in the DMSO-treated group (Fig. 3C, $P < 0.05$). We further evaluated the subpopulations of CD4 T lymphocytes as well as the proportions of IFN-γ-producing CD4 cells (Th1 cells), IL-4-producing CD4 cells (Th2 cells) and regulatory T cells (Treg cells). A significant reduction was observed in the percentage of the Th1 subpopulation (Fig. 3D, $P < 0.05$), while no significant difference in IL-4-producing CD4 cells (Th2 cells) was found (Fig. 3E). However, the CD4 CD25 Foxp3 cell (Treg cell) population was significantly increased in the DMSO-treated group ($P < 0.05$) (Fig. 3F, $P < 0.05$). We also analyzed the expression of IFN-γ and IL-4 cytokines in the splenocytes (Figs. 4A and B) of PBS or DMSO-treated NOD mouse recipients via real-time RT-PCR. The levels of IFN-γ were significantly decreased with DMSO treatment. However, the levels of IL-4 cytokine displayed no difference between the DMSO-treated and PBS-treated groups. These results suggest that the extended survival of syngeneic islet grafts in recipients may be attributed to the DMSO-mediated decreases in CD8, DC and Th1 cells, which is likely acting via modulation of Treg cells in the experimental mice.

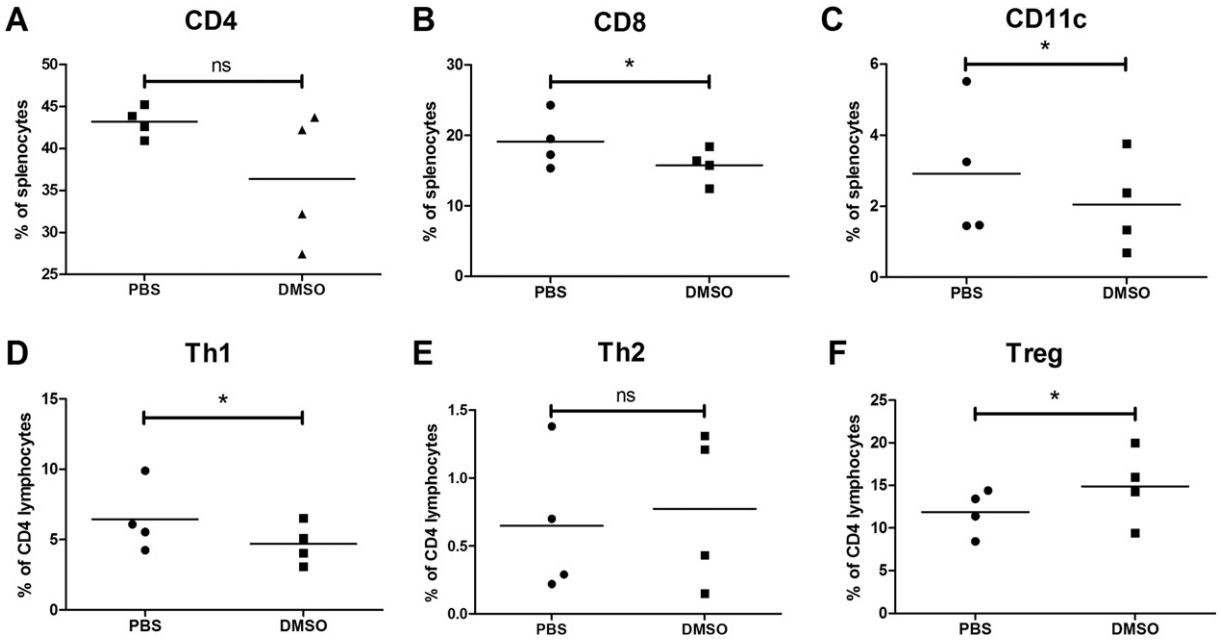

Fig. 3. DMSO treatment reduces the population of CD8 cells, Th1 cells and DCs, and increases Treg cells in the spleen of NOD mice. The (A) CD4 T lymphocytes, (B) CD8 T lymphocytes, (C) DCs, (D) Th1 cells, (E) Th2 cells and (F) Treg cells were isolated from the spleens of DMSO-treated and PBS-treated NOD recipients at day 8 post-transplantation and then analyzed via flow cytometry. $^{*}p < 0.05$. ($n = 4$).

The proliferation of T cells was inhibited by DMSO treatment

To test whether the proliferative capacity of splenocytes in grafted recipients was reduced by DMSO treatment, we evaluated the proliferation of splenocytes via ^{3}H-thymidine incorporation assay. Islet-transplanted NOD mice were treated with DMSO (at 0.22 g/kg/day for 9 days) or PBS for 9 days; splenocytes were then harvested and stimulated with α-CD3 (0.25, 0.5, 1, and 2 μg/mL) and Con A (0.25, 0.5, 1, and 2 μg/mL), respectively. The proliferation of splenocytes harvested from the DMSO-treated mice was significantly reduced compared with the

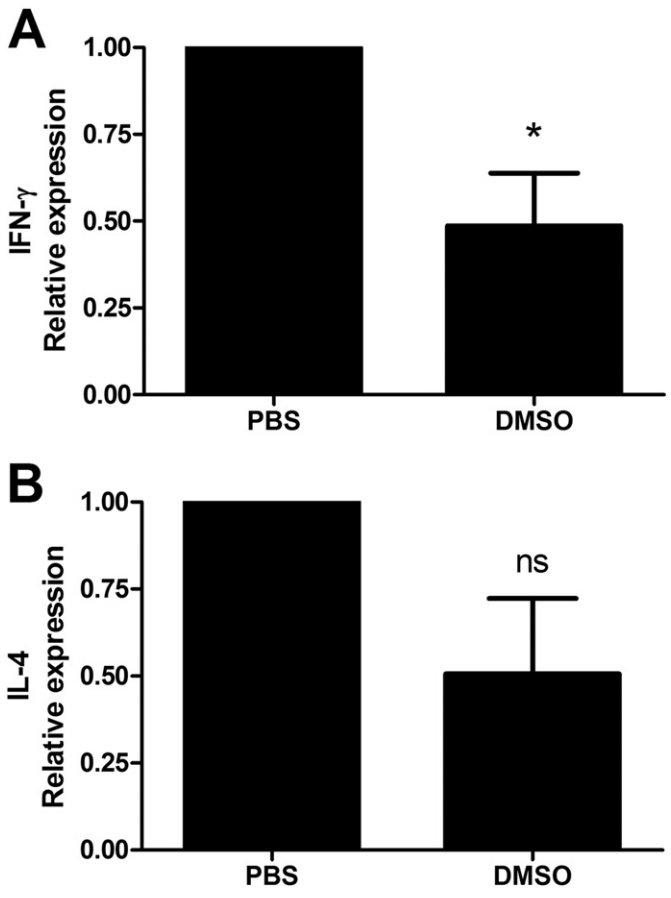

Fig. 4. DMSO treatment reduces IFN-γ cytokine expression in the splenocytes of NOD mice. The (A) IFN-γ and (B) IL-4 cytokine expression levels in splenocytes isolated from DMSO-treated or PBS-treated regular NOD female mice were analyzed via real time RT-PCR. $^{*}p < 0.05$. The data are expressed as the mean ± SEM ($n = 3$).

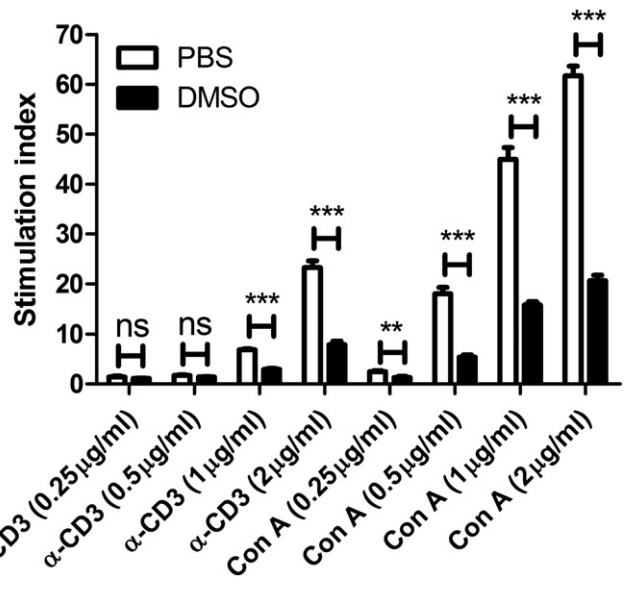

Fig. 5. DMSO treatment inhibits the proliferation of T lymphocytes in islet-transplanted NOD mice. The islet-transplanted NOD mice were treated with DMSO or PBS for 9 days. Splenocytes were harvested and stimulated with α-CD3 (0.25, 0.5, 1, and 2 μg/mL) and Con A (0.25, 0.5, 1, and 2 μg/mL). The number of splenocytes harvested from DMSO-treated mice was significantly reduced compared with the PBS-treated group ($^{*}p < 0.05$). The data are expressed as the mean ± SEM ($n = 3$).

proliferation of splenocytes harvested from the PBS-treated group (Fig. 5). These results suggest that the proliferative ability of T cells in the splenocytes of NOD recipients was inhibited by DMSO treatment.

DMSO treatment induced Treg differentiation from naive CD4 T cells

To investigate whether the protective effect of DMSO treatment acts via the induction of Treg cell differentiation, we examined the ability of Treg cell differentiation from naive CD4 T cells in NOD recipients with DMSO treatment. These naive CD4 T cells were cultured in different concentrations of DMSO-containing medium (0%, 0.5% and 1%) at various time periods (0, 6, and 12 h). The percentages and absolute numbers of Treg cells differentiated from these naive CD4 T cells were assessed via flow cytometry. The percentages and absolute numbers of Treg cells were significantly increased following DMSO treatment in a dose-dependent manner (Figs. 6A and B). We also examined the influence of DMSO treatment in the activation of the STAT5 signaling pathway, which is a critical factor in Treg cell differentiation (Burchill et al., 2007). The expression levels of STAT5 and the phosphorylation of STAT5 (STAT5-P) were increased following DMSO treatment in a time dependent manner (Fig. 7). These results suggest that the protective effect observed with DMSO treatment in syngeneic islet transplantations may be attributed to the DMSO-mediated modulation of Treg cell differentiation in recipients.

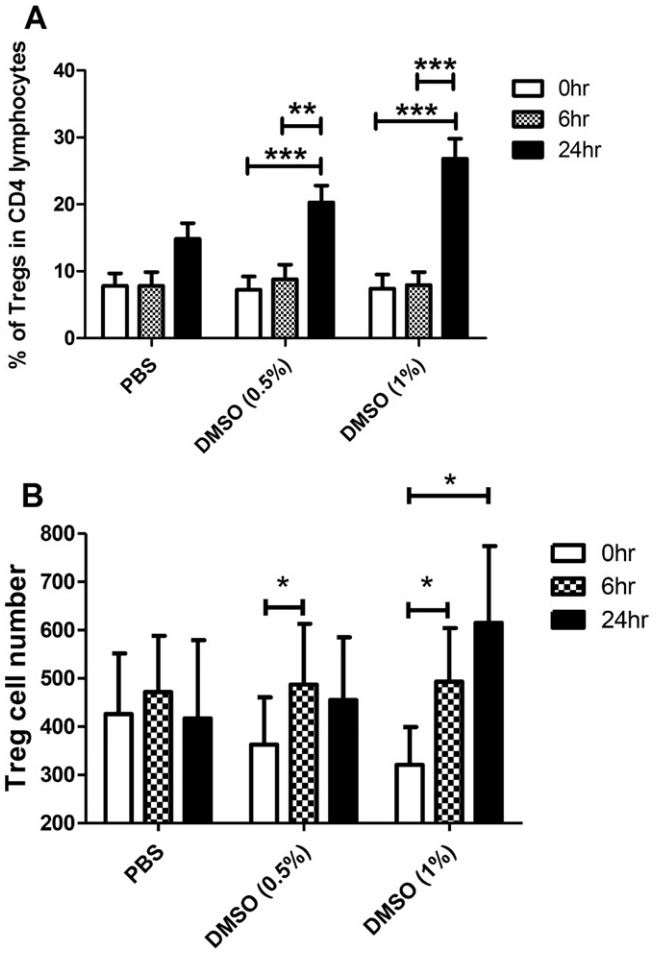

Fig. 6. DMSO treatment increased the differential population and cell number of Treg lymphocytes in the naive CD4 T lymphocytes of NOD mice. Naive CD4 T lymphocytes were cultured with differential concentrations of DMSO solutions (0%, 0.5% and 1%) for different incubation times (0 h, 6 h and 24 h). After DMSO solution treatment, the percentage and number of Treg lymphocytes were analyzed via flow cytometry. The (A) percentages and (B) numbers of Treg cells differentiated from naive CD4 T lymphocytes following DMSO treatment at different concentrations and incubation times were increased ($^{*}p < 0.05$, $^{**}p < 0.01$, , $^{***}p < 0.001$). The data are expressed as the mean ± SEM ($n = 6$).

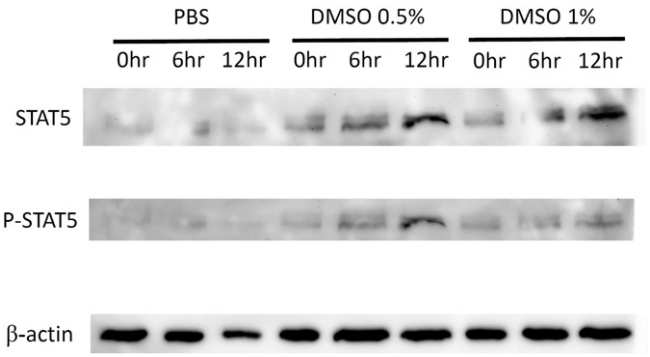

Fig. 7. DMSO treatment increased STAT5 and phosphorylated STAT5 levels in CD4 T lymphocytes of NOD mice. The CD4 naive T lymphocytes were cultured with various concentrations of DMSO solutions (0%, 0.5% and 1%) at different incubation times (0 h, 6 h and 12 h). After DMSO solution treatment, the levels of STAT5 and phosphorylated STAT5 (P-STAT5) were assessed via western blot. The result is representative of three independent experiments.

Discussion

T1D is an autoimmune disease caused by the destruction of islet β cells by autoreactive immune cells (Kawasaki et al., 2004). However, most T1D patients are diagnosed after diabetes onset. Islet transplantation has been attempted as an effective therapy strategy for T1D (Bottino et al., 2002) due to the lower cost and higher safety compared with pancreas transplantation (Robertson, 2004; Gaglia et al., 2005). However, recurrent autoimmunity plays a crucial role in islet graft destruction and impairs the survival of islet grafts in human islet transplantation. Moreover, destruction of grafts by autoimmune recurrence often takes place earlier than allogeneic graft rejection (Balamurugan et al., 2006; Huang et al., 2013). Therefore, it is important to overcome recurrent autoimmunity in islet transplantation.

DMSO is a frequently used solvent for organic or inorganic substances. It also exhibits therapeutic benefits, including anti-inflammation and anti-ROS effects, in the treatment of several human diseases, such as interstitial cystitis and rheumatoid arthritis (Kato et al., 2000; Santos et al., 2003). Recently, the immune-modulatory effect of DMSO has been reported by Henrik Kloverpris et al. (2010). Human T cell line responses are completely abrogated by DMSO treatment at concentrations between 2.5% and 5% for 6 h (Kloverpris et al., 2010). In this study, we investigated the protective effects of DMSO treatment on the prevention of spontaneous diabetes and the inhibition of autoimmune recurrence in islet graft rejection with a safe dosage. This is the first report showing that DMSO treatment effectively suppresses the onset of autoimmune diabetes and prolongs the survival of islet grafts. Our results also demonstrate that DMSO treatment reduces the population of dendritic cells and Th1 cells among the splenocytes of NOD mice. Furthermore, we also found that this treatment increased the percentages of Treg cells among splenocytes, thereby suggesting that DMSO exerts an inductive effect in the generation of Treg cells. This increase in Treg cells may explain the suppressive effect of DMSO on the population of Th1 cells as well as the production of IFN-γ.

We further examined whether DMSO treatment induces the generation of Treg cells. We found that DMSO treatment induced the differentiation of Treg cells from naive CD4 T lymphocytes, thereby demonstrating a novel effect of DMSO in the production of Treg cells. Therefore, we further investigated the underlying mechanism in the increase of Treg cells induced by DMSO. A previous study has reported that the STAT5 signaling pathway is required for the development of Treg cells (Burchill et al., 2007). A recent study has also demonstrated that STAT5 polarization promotes Treg cell generation (Betts et al., 2014). These results highlight the

importance of the STAT5 signaling pathway in the production of Treg cells. Therefore, we investigated whether DMSO-induced Treg differentiation acts via enhancement of the activation of the STAT5 signaling pathway. We found that the levels of STAT5 protein and phosphorylated STAT5 were increased following DMSO treatment, thereby suggesting that DMSO may promote differentiation of Treg cells via enhanced the activation of the STAT5 signaling pathway.

A major concern in medicinal therapy for the modulation of immune responses is the safety of drug administration. DMSO has been demonstrated to elicit minor side effects in the clinical setting (Jacob and de la Torre, 2009). The best known adverse reaction is intravascular hemolysis after intravenous infusion of DMSO solution with concentrations equal to or greater than 40% (Waller et al., 1983). This could be due to the osmotic pressure in erythrocytes caused by DMSO (Wolf and Simon, 1983), which can be prevented by using DMSO solution at less than 30% (Karaca et al., 2002). Our data indicate that DMSO treatment at the dosage of 0.22 g/kg/day in NOD mice was safe and yielded no obvious diarrhea or weight loss. Furthermore, this treatment did not interfere the metabolism of blood glucose and the insulin-secreting ability of pancreatic cells in treated NOD mice. Compared with DMSO, the side effects of Tacrolimus (FK506), which is a calcineurin blocker for T cells frequently used in the inhibition of allograft rejection, include the induction of hyperglycemia or diabetes in recipients after human islet transplantation (Nanji and Shapiro, 2004). This effect may alter the insulin-secreting function of β cells in the islet by suppressing the mRNA expression of insulin gene 1 and 2 (Hernandez-Fisac et al., 2007). Furthermore, FK506 also affects glucose-induced insulin secretion by interacting with the exocytotic machinery of the β cell. It has been shown that dephosphorylation of kinesin heavy chain by the Ca^{2+}-dependent protein phosphatase 2B/calcineurin might be required for normal stimulation of the second (microtubule-dependent) phase of glucose-induced insulin secretion (Heit et al., 2006). Thus, DMSO may be an alternative therapeutic option for islet transplantation in the therapy of T1D.

In conclusion, our study demonstrated for the first time that DMSO exerts therapeutic effects in the inhibition of autoimmune recurrence in islet transplantation for T1D treatment. DMSO acts through the immune modulatory effect by suppressing the Th1 immune response and inducing Treg cell differentiation by enhancing the activation of the STAT5 signaling pathway.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgments

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